WideScreen™ Breast Cancer Panel I, II and III

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About the Kits

Cell Extraction Kit	1 kit	71926-3
WideScreen™ Reagent Kit	1 kit	71783-3
WideScreen Breast Cancer Panel I, II, and III Kits		See Appendix A

Overview

Bead-based flow cytometric xMAP® assays enable sensitive, precise quantitation of analytes within a sample. When directed toward proteins, such assays are essentially sandwich immunoassays on a bead. Samples are combined with fluorescently labeled microparticles (beads) covalently conjugated to a capture antibody. Analytes captured on the beads are identified with detection antibodies and a fluorescent label. A major hallmark of bead-based assays over traditional protein quantitation methods (such as ELISA) is the capacity for multiplexing, as bead-based assays allow simultaneous quantitation of multiple analytes in a small sample volume. Other advantages of xMAP assays include flexibility, robustness, simple sample handling, and requirement of minimal sample volumes, making them an ideal platform for immunodetection of biomarkers and signaling proteins.

WideScreen™ Breast Cancer Panels I, II, and III are premixed multiplex bead kits for simultaneous quantitation of proteins reported to be involved in breast cancer (see Table 1 for a complete list of analytes) The bead kits are available as a choice of three multiplex panels and individually, facilitating the creation of singleplex and custom multiplex experiments. WideScreen Breast Cancer Panel I, II and III Complete Kits include all the reagents and buffers needed to analyze the proteins listed below each respective panel (see Table 1) in cell culture lysates and normal or tumor tissue lysates using the Luminex® xMAP® System.

Table 1: Target recognition for Breast Cancer Panels I, II, and III

Breast Cancer Panel I	Breast Cancer Panel II	Breast Cancer Panel III
Angiopoietin-2	EGFR	TIMP-1
Progesterone receptor	Fas	uPA
HER-2	VEGFR-2	E-cadherin
PAI-1	Estrogen receptor α	IGFBP-3
IGF-1R	TIMP-2	
Angiogenin		

Species specificity: Human, other species not tested.

For more information on Widescreen Bead-based Assays, visit www.novagen.com/WideScreen.

Breast Cancer Panel I, II, and III analytes are directly or indirectly implicated in breast cancer biology including tumor growth, apoptosis, angiogenesis, and metastasis. These include receptor proteins and a myriad of signaling and effector proteins that have the ability to “crosstalk” with each other. The levels of many of these proteins in breast tumor tissue differ when compared to adjacent, normal tissue. Identifying these proteins can assist in the understanding of the tumor microenvironment and the fundamental biology of tumor formation.

Expression profiling of extracellular receptor tyrosine kinases such EGFR and HER-2 and intracellular steroid receptors such as estrogen receptor α, and progesterone receptor in breast tumor tissue has resulted in tumor classification that aids in breast cancer treatment and prevention. Another receptor tyrosine kinase, IGF-1R, is known to promote growth, transformation and antiapoptotic activity of breast tumor tissue. IGF-1R activity is further controlled by binding proteins such as IGFBP-3 that bind to IGFs with a greater affinity compared to the IGF-1R receptor. Additional signaling molecules are involved in controlling the apoptosis of the breast cancer cells. For instance, the expression levels of Fas receptor and its ligand FasL on breast tumor tissue regulate Fas-mediated apoptosis in breast cancer cells.

Breast tumor growth is promoted by proteins that are involved in new blood vessel formation, i.e., angiogenesis. A primary player is the receptor protein VEGFR-2 that is expressed on breast tumor tissue with its ligand VEGF-A. Other pro-angiogenic molecules involved in breast cancer include: angiogenin,

inducing neovascularization, and angiopoietin-2, affecting vascular remodeling via integrin and E-cadherin signaling pathways.

While vascular remodeling is important for solid tumor growth, it also contributes to the spreading of the primary tumor to secondary sites: metastasis, a leading cause of poor breast cancer prognosis. Several extracellular matrix remodeling activities are required for tumor invasion and metastasis. An example of a proteolytic protein involved in breast cancer invasion and metastasis is urokinase-type plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1) that affect fibrinolysis and cell migration. In addition, proteolytic enzymes such as matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs) mediate extracellular matrix remodeling. Specifically, MMP-9:TIMP-1 and MMP-2:TIMP-2 ratios have been shown to regulate the invasive potential of breast tumors.

Components

WideScreen™ Breast Cancer Panel Bead Kits are used for quantitative, multiplex analysis of cell culture lysates and normal or tumor tissue lysates. The WideScreen Breast Cancer Panel I, II, and III Complete Kits contain sufficient reagents to assay one 96-well plate. For maximum flexibility and user-defined multiplex assay configuration, the components of the WideScreen Breast Cancer Panel I, II, and III Complete Kits are available separately.

WideScreen Breast Cancer Panel I, II and III Complete Kits

The WideScreen Breast Cancer Panel I, II, or III Complete Kits include the entire set of reagents to assay one 96-well plate, including the Breast Cancer Panel I, II or III multiplex, Breast Cancer Panel I, II or III Standards Mix, Cell Extraction Kit, and WideScreen Reagent Kit.

Breast Cancer Panel I, II and III

Breast Cancer Panel I, II and III are premixed panels that contain premixed antibody-coated Capture Beads, biotinylated Detection Antibodies and premixed recombinant standards used for target quantification via multiplex sandwich immunoassay. Each Breast Cancer Panel contains sufficient reagents to assay one 96-well plate.

Note: Breast Cancer Panel I, II and III require the purchase of the Cell Extraction kit and the WideScreen Reagent Kit.

Target analytes for the premixed panels are listed in Table 1, *Appendix A* on p 21 and at www.novagen.com/WideScreen.

Each premixed standards mix contains reagents sufficient to generate eight multiplex standard curves, or four standard curves in duplicate. The concentration of premixed standards in the standard curves can be found in *Appendix B* on p 23 and at www.novagen.com/WideScreen. Performance specifications for the Multiplex Kits are detailed in the Certificates of Analysis, available online.

Individual Breast Cancer Panel I, II and III Bead Kits

Individual Bead Kits for each analyte for setting up singleplex or user-defined multiplex experiments may be purchased separately. These kits include antibody-coated Capture Beads, biotinylated Detection Antibody and the appropriate individual recombinant standard used for target detection via sandwich immunoassay. Each individual Bead Kit contains sufficient reagents to assay one 96-well plate.

Note: Individual Bead Kits require the purchase of the Cell Extraction kit and the WideScreen Reagent Kit.

Available Individual Bead Kits are listed in *Appendix A* on p 21 and at www.novagen.com/WideScreen.

The concentration of each standard can be found in *Appendix B* on p 23 and at www.novagen.com/WideScreen. Each individual standard contains reagents sufficient to generate eight singleplex standard curves, or four standard curves in duplicate. Performance specifications for the Individual Bead Kits are detailed in the individual Certificates of Analysis, available online.

Note: Individual Breast Cancer Panel Bead Kits and buffers are not necessarily compatible with other bead kits and reagents sold by Novagen or other vendors. Please refer to Appendix C on p 25 for compatibility of assays found in WideScreen Breast Cancer Panels I, II, and III.

Cell Extraction Kit

The Cell Extraction Kit contains a cell extraction reagent that releases soluble and membrane proteins efficiently. Benzonase® Nuclease (included) reduces viscosity due to chromosomal DNA. Phosphatase and protease inhibitor cocktails (included) maintain the phosphorylation state and integrity of target proteins during cell extraction. The kit contains reagents sufficient to make 20 ml of cell lysate or to process 160 wells of cells grown in 96-well plates. Additional extraction reagent is included for preparation of titration buffer.

WideScreen™ Reagent Kit

The WideScreen™ Reagent Kit contains reagents needed for the bead-based immunoassays, including all buffers, a 96-well Filter Plate, a Plate Sealer, and a Streptavidin-Phycoerythrin solution used in the final detection step. The kit contains sufficient reagents to perform 96 singleplex or multiplex bead-based tests.

Components and Storage

Cell Extraction Kit		**71926-3**
25 ml	Extraction Reagent	Store at –20°C
500 µl	Phosphatase Inhibitor Cocktail Set V (50x)	Store at –20°C
25µl	Protease Inhibitor Cocktail Set III (1000x)	Store at –20°C
10 µl	Benzonase® Nuclease HC, Purity >99% (250 U/µl)	Store at –20°C

WideScreen™ Reagent Kit		**71783-3**
100 µl	Streptavidin-PE Concentrate	Store at 4°C
20 ml	10X Wash Buffer	Store at 4°C
25 ml	5X Assay Diluent	Store at 4°C
1 ea	Polyethylene Plate Sealer	Store at room temperature
1 ea	96-well Filter Plate	Store at room temperature

Components and storage conditions for WideScreen Breast Cancer Panel Complete Kits and Breast Cancer Panel I, II, and III Multiplex and Individual Bead Kits are described in *Appendix A* on p 21.

Additional Materials Required But Not Supplied

- Experimental samples, such as cultured cell lines or tissue samples
- Dounce Homogenizer or Micro-dismembrator (for preparing tissue lysates)
- Luminex® System (or comparable, such as Bio-Plex® Suspension Array System)
- xMAP data analysis software (e.g., Luminex IS™, ACS StarStation, Bio-Plex Manager™, or comparable)
- Vacuum manifold for filter plates (Pall 5017 or Millipore Cat. No. MAVM0960R)
- 96-well plate platform shaker, such as IKA MTS4
- BCA protein assay kit (Novagen® Cat. No. 71285)
- Polypropylene microcentrifuge tubes
- 15 ml and 50 ml polypropylene centrifuge tubes
- Microcentrifuge
- Vortexer
- Ultrasonic bath, such as Cole Parmer EW-08849 (optional)
- Multichannel pipet (optional)
- Fixation solution (0.2% paraformaldehyde in PBS) (optional)
- Syringe-tip filter (0.45 µm) and syringe, or 96-well filter plate (e.g. Millipore Cat. No. MSDVN6510) and 96-well collector plate
- Tris-buffered saline (TBS) (10 mM Tris, pH 7.5, 150 mM NaCl)

Growth of Cell Lines

Considerations Before You Begin

- Growth rate and requirements for optimal growth vary considerably between cell lines; even the same cell line will grow differently in different laboratories. The following conditions are intended as general guidelines only.
- Cells maintained in culture for long periods of time tend to exhibit slower growth rates and become refractory to stimulation conditions. In general, cell lines passaged <15 times are recommended.

Protocol for Growth of Cell Lines

1. Culture cells in T-75 flasks until steady growth is established. Most cell lines will tolerate a split of 1:10–1:20 without slowing their growth rate.
2. Culture adherent cells until they approach a confluent monolayer, or suspension cells until they approach 10^6 cells per ml. Slower-growing cell lines (such as A431) may initially take up to a week to approach confluency.
3. Plate cells, using the following table as a general guide. Harvest cells for lysate preparation after 2 or 3 days, depending on whether the cells are serum starved overnight before harvesting.

Table 2. Approximate Cell Numbers for Seeding Cell Lines

Cell Line	T-75 Flask or 10 cm Dish	6-well Plate (per well)	96-well Plate (per well)
T47D	2.4×10^6	3.4×10^5	3.0×10^4
MCF-7	1.2×10^6	1.7×10^5	1.5×10^4
BT20	1.2×10^6	1.7×10^5	1.5×10^4
A431	2.0×10^6	2.8×10^5	4.0×10^4
HeLa	1.2×10^6	1.7×10^5	1.5×10^4
HepG2	4.8×10^6	6.8×10^5	8.0×10^4
HT29	2.4×10^6	3.4×10^5	3.0×10^4
HUVEC	1.5×10^5 *	2.0×10^5	not recommended
NHDF	1.5×10^5 *	1.5×10^5	not recommended
SK-Br-3	2.0×10^6	2.8×10^5	3.0×10^4
Jurkat	1.0×10^6	1.4×10^5	1.5×10^4

* HUVEC and NHDF cells plated with these cell numbers are serum-starved after 6 days and lysed after 7 days.

Note: If cells are grown in 96-well plates, plate extra wells for determining total protein concentration of the lysates.

4. Prepare lysates when cell density is high, but cells are still growing logarithmically. For adherent cells, this is typically a monolayer that is ~ 80% confluent. For suspension cells, this is typically a density of 0.5–1.0 x 10^6 per ml.

Lysate Preparation

Considerations Before You Begin

- Do not omit steps from the sample preparation protocol. All steps are necessary for optimum assay performance.

- If it is important to know the lysate protein concentration from cells grown in 96-well plates, prepare additional wells of cells solely for this purpose.
- If using cells grown in 96-well plates, avoid plating cells in the outermost wells. This minimizes cell growth edge effects.

Lysis Protocol for Cell Lines

1. Prepare 1X Assay Diluent by adding 25 ml 5X Assay Diluent (WideScreen™ Reagent Kit) to 100 ml sterile distilled deionized water. Store 1X Assay Diluent that will be used within one month at 4°C. To avoid microbial growth, dispense working aliquots of any remaining 1X Assay Diluent and store at –20°C.
2. Prepare 1X Wash Buffer by adding 20 ml 10X Wash Buffer (WideScreen Reagent Kit) to 180 ml sterile distilled deionized water. Store at 4°C.
3. Calculate the total amount of Extraction Reagent needed. Prepare 10% excess to account for pipetting error.

Format	Extraction Reagent
T-175 flask	4 ml
T-75 flask	2 ml
T-25 flask	1 ml
6-well	200 µl/ well
96-well	120 µl/ well

4. Prepare the required volume of supplemented Extraction Reagent:

Per ml Extraction Reagent, add:

1 µl	Protease Inhibitor Cocktail III (1000X)
0.1 µl	Benzonase® Nuclease
20 µl	Phosphatase Inhibitor Cocktail Set V (50X)

Notes: *Prepare fresh supplemented Extraction Reagent each time cell lysates are made.*

Because the Breast Cancer Panel assays are not phospho-specific, the use of Phosphatase Inhibitor Cocktail Set V is optional.

5. Aspirate and discard culture medium.
6. On ice, rinse cell monolayer twice with cold Tris-buffered saline (TBS). Remove all TBS.
   For non-adherent cells: transfer cells to centrifuge tubes, centrifuge at 500 x *g*, and wash twice with ice-cold TBS.
7. Add cold supplemented Extraction Reagent to adherent cells. Incubate for 20 min at 4°C with gentle agitation (rocking platform or occasional swirling).
   For non-adherent cells, tap the centrifuge tube with finger to loosen cell pellet. Add supplemented Extraction Reagent. Incubate for 20 min at 4°C with occasional vortexing.
8. Dislodge and solubilize all adherent cells using a rubber policeman or by repeated pipeting. Extracts should be clear and non-viscous.
9. Clear lysates by filtration. Pre-wet filter or filter plate with TBS, then remove all excess buffer. For lysates with volume >0.2 ml, use syringe-tip filter (pore size 0.45 µm). For lysates with volume < 0.2 ml, use a 96 well-filter plate (e.g. Millipore Cat. No. MSDVN6510, and centrifuge at 1500 x *g* for 1 min at 4°C. Place a 96-well plate under the filter plate during centrifugation to collect lysates.)
10. Remove a 50 µl sample of each extract for protein quantification by BCA Protein Assay (Cat. No. 71285). Determine the total protein concentration of each extract.

Note: *Typical total protein concentrations from cells grown in flasks range from 0.4 mg/ml to 2 mg/ml, depending on the cell line and confluence. Typical total protein concentrations from cells grown in 96-well plates range from 0.1– 0.5 mg/ml.*

11. Either proceed immediately to the Bead-based Immunoassay Protocol on p 10, or store aliquots at –70°C. Avoid multiple freeze-thaw cycles.

Lysis Protocol for Tissue Samples

Tissue homogenization and lysate preparation using Dounce Homogenizer

For this protocol, a Dounce Homogenizer is recommended. Fresh or flash-frozen tissue samples can be used. This protocol has been successfully applied to breast tissue, using a 1:15 (w/v) ratio of tissue to supplemented Extraction Reagent. Other tissue types may require ratio optimization. For homogenization, use a tube large enough to accommodate some foaming.

1. Calculate the total amount of Extraction Reagent needed. Prepare 10% excess to account for pipetting error.
2. Prepare the required volume of supplemented Extraction Reagent:

Per ml Extraction Reagent, add:	
1 µl	Protease Inhibitor Cocktail III (1000X)
0.1 µl	Benzonase® Nuclease
20 µl	Phosphatase Inhibitor Cocktail Set V (50X)

Notes: *Prepare fresh supplemented Extraction Reagent each time tissue lysates are made.*

Because the Breast Cancer Panel assays are not phospho-specific, the use of Phosphatase Inhibitor Cocktail Set V is optional.

3. Determine tissue weight. Transfer aliquots of 50–120 mg tissue to 5 ml round-bottom polypropylene tubes. Keep samples on ice (normal tissue ~120 mg; tumor tissue ~50 mg).
4. Add 15 parts (v/w) room temperature supplemented Extraction Reagent for each part tissue (e.g., 750 µl supplemented Extraction Reagent per 50 mg tissue) Immediately return sample to ice.
5. Homogenize with a hand-held homogenizer or other suitable device. Homogenization time will vary with tissue type, but ~1 minute is generally required for efficient protein extraction. Return sample to ice.
6. Incubate for 30 min on ice with occasional vortexing.
7. Clarify lysate by centrifuging twice at 16,000 x g for 10 min at 4°C. After each centrifugation, transfer supernatants to new microcentrifuge tubes after each centrifugation step.
8. Remove a 50 µl sample of each extract for protein quantification by BCA Protein Assay (Cat. No. 71285). Determine the total protein concentration of each extract.
9. Either proceed immediately to the Bead-based Immunoassay Protocol on p 10 , or store aliquots at –70°C. Avoid multiple freeze-thaw cycles.

Tissue homogenization and lysate preparation using Micro-dismembrator

For this protocol, a Micro-dismembrator (bead or ball mill) is recommended. Flash-frozen tissue samples should be used. This protocol has been successfully applied to breast tissue, using a 1:10 (w/v) ratio of tissue to Supplemented Extraction Reagent. Other tissue types may require ratio optimization.

1. Prepare the required volume of supplemented Extraction Reagent:

Per ml Extraction Reagent, add:	
1 µl	Protease Inhibitor Cocktail III (1000X)
0.1 µl	Benzonase Nuclease
20 µl	Phosphatase Inhibitor Cocktail Set V (50X)

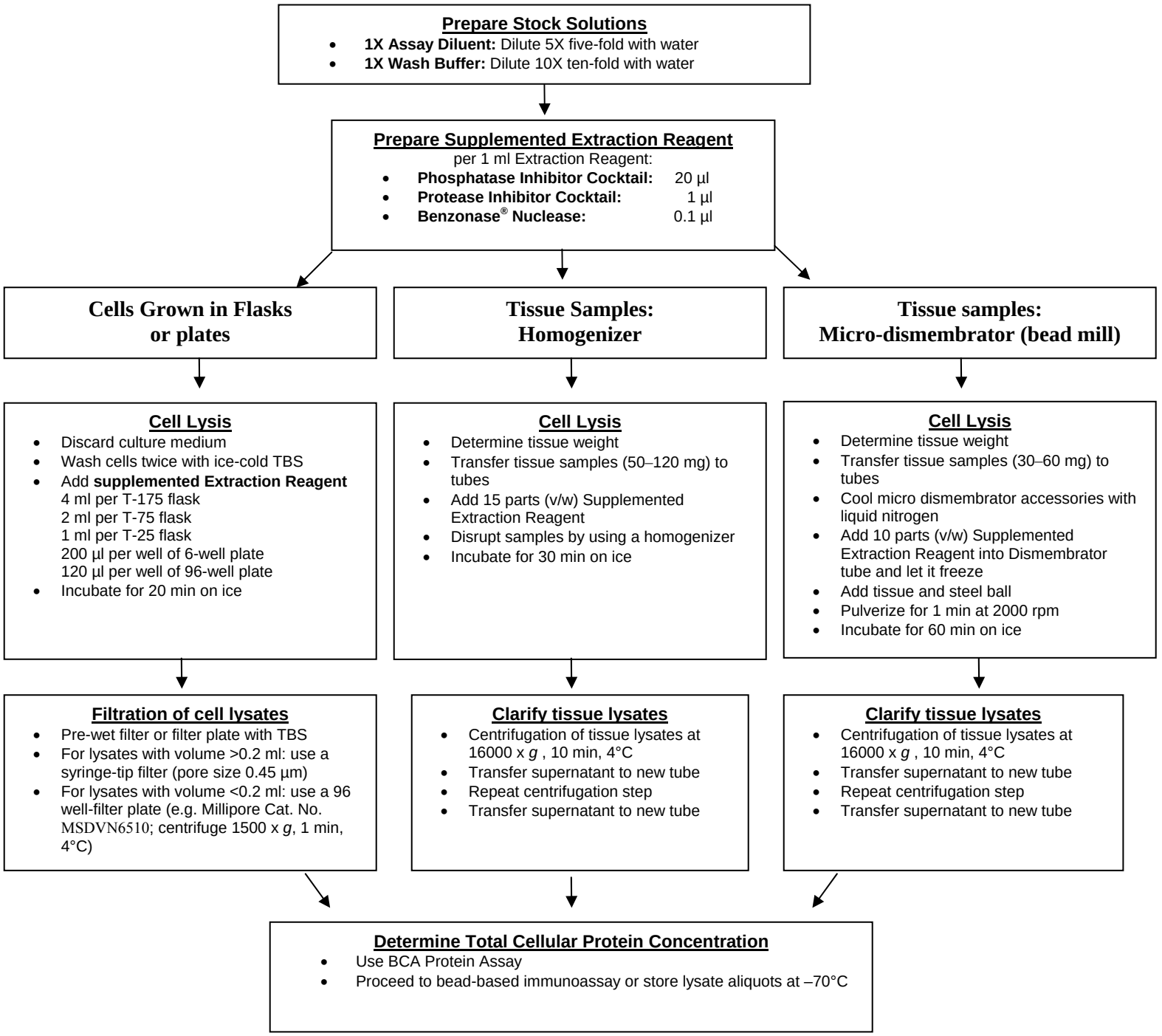
Notes: *Prepare fresh supplemented Extraction Reagent each time cell lysates are made.*

Because the Breast Cancer Panel assays are not phospho-specific, the use of Phosphatase Inhibitor Cocktail Set V is optional.

2. Determine tissue weight. Transfer aliquots of 30–60 mg tissue to a tube and keep samples frozen (normal tissue ~60 mg; tumor tissue ~30 mg).
3. Freeze accessories for Micro-dismembrator with liquid nitrogen.
4. Add 10 parts Supplemented Extraction Reagent for each part tissue (e.g., 500 µl supplemented Extraction Reagent per 50 mg tissue) into the dismembrator tube and let it freeze.
5. Add tissue and steel ball, close the tube and pulverize for 1 minute at 2000 rpm.
6. Incubate for 60 min on ice with occasional vortexing.

7. Clarify lysate by centrifuging twice at 16,000 x *g* for 10 min at 4°C. After each centrifugation, transfer supernatants to new microcentrifuge tubes after each centrifugation step.
8. Remove a 50 µl sample of each extract for protein quantification by BCA Protein Assay (Cat. No. 71285). Determine the total protein concentration of each extract.
9. Either proceed immediately to the Bead-based Immunoassay Protocol on p 10, or store aliquots at –70°C. Avoid multiple freeze-thaw cycles.

Flowchart for Lysate Preparation


Prepare Stock Solutions
1X Assay Diluent: Dilute 5X five-fold with water
1X Wash Buffer: Dilute 10X ten-fold with water
Prepare Supplemented Extraction Reagent
per 1 ml Extraction Reagent:
Phosphatase Inhibitor Cocktail: 20 µl
Protease Inhibitor Cocktail: 1 µl
Benzonase® Nuclease: 0.1 µl
Cells Grown in Flasks or plates
Tissue Samples: Homogenizer
Tissue samples: Micro-dismembrator (bead mill)
Cell Lysis
Discard culture medium
Wash cells twice with ice-cold TBS
Add supplemented Extraction Reagent
4 ml per T-175 flask
2 ml per T-75 flask
1 ml per T-25 flask
200 µl per well of 6-well plate
120 µl per well of 96-well plate
Incubate for 20 min on ice
Cell Lysis
Determine tissue weight
Transfer tissue samples (50–120 mg) to tubes
Add 15 parts (v/w) Supplemented Extraction Reagent
Disrupt samples by using a homogenizer
Incubate for 30 min on ice
Cell Lysis
Determine tissue weight
Transfer tissue samples (30–60 mg) to tubes
Cool micro dismembrator accessories with liquid nitrogen
Add 10 parts (v/w) Supplemented Extraction Reagent into Dismembrator tube and let it freeze
Add tissue and steel ball
Pulverize for 1 min at 2000 rpm
Incubate for 60 min on ice
Filtration of cell lysates
Pre-wet filter or filter plate with TBS
For lysates with volume >0.2 ml: use a syringe-tip filter (pore size 0.45 µm)
For lysates with volume <0.2 ml: use a 96 well-filter plate (e.g. Millipore Cat. No. MSDVN6510; centrifuge 1500 x g, 1 min, 4°C)
Clarify tissue lysates
Centrifugation of tissue lysates at 16000 x g , 10 min, 4°C
Transfer supernatant to new tube
Repeat centrifugation step
Transfer supernatant to new tube
Clarify tissue lysates
Centrifugation of tissue lysates at 16000 x g , 10 min, 4°C
Transfer supernatant to new tube
Repeat centrifugation step
Transfer supernatant to new tube
Determine Total Cellular Protein Concentration
Use BCA Protein Assay
Proceed to bead-based immunoassay or store lysate aliquots at –70°C

Bead-based Immunoassay Protocol

Considerations Before You Begin

- Have on hand the 1X Assay Diluent and 1X Wash Buffer that was prepared during the Lysate Preparation protocol.
- Important guidelines to follow when using filter plates and the vacuum manifold:
    - Excessive vacuum will cause the filter plate membrane to perforate. Adjust the manifold using a non-filter (ELISA or tissue culture) plate, ensuring that the vacuum cannot exceed 5 in (127 mm) Hg.
    - After adjusting the vacuum, place filter plate on the manifold. Use fingertips to apply pressure evenly across the plate. The liquid should drain in 2–5 sec.
    - To avoid drying out the beads, vacuum only long enough to drain all wells. Do not allow drained beads to sit for more than 1 min before rehydrating with buffer.
    - It is critical to remove excess buffer from the underside of the filter plate by tapping it on a paper towel several times before adding samples or reagents. This prevents samples from wicking out of the wells during incubation steps. For the same reason, avoid placing filter plate on an absorbent surface during incubations.
    - To avoid perforating the filter plate membrane, be sure that the probe height on the Luminex® System is adjusted correctly. Do not touch the membrane with pipet tips. For accurate pipetting, touch tips to the sides of the filter plate wells. Change tips as necessary to prevent cross-contamination.
- Capture Beads contain fluorescent dyes and are therefore light-sensitive. To avoid photobleaching, keep beads in microcentrifuge tubes covered. Cover filter plates containing beads with aluminum foil during incubation steps. Streptavidin-PE solution is also light-sensitive; protect from light.
- To prevent fluorescent dye loss, do not use organic solvents with capture beads. Beads are incompatible with DMSO concentrations >1%.
- Many of the liquid handling steps such as washing are done most easily with an 8-channel or 12-channel pipet (manual or automatic). However, for best results, use accurate single-channel pipets for manipulation of standards and experimental samples.
- If using multichannel pipets, ensure that tips fit correctly. Verify volume accuracy and consistency.
- To conduct the protocol efficiently, prepare reagents for the next step during sample incubation period.
- When calculating the amount of reagents needed during the various steps, prepare 10% excess to allow for pipetting error.
- Run standard dilution series and experimental samples using the same multiplex configuration. For instance, if a 6-Plex of Bead Kits is used to measure experimental samples, the same 6-Plex should be used to create the standard dilution series. Multiplexing causes slight shifts in some standard curves, which will make quantification inaccurate unless experimental samples are measured using the same multiplex.
- For best overall assay performance, lysates are diluted at least 4-fold when incubating with the Capture Beads. If desired, lysates can be tested at a 2-fold final dilution, although this concentration of Lysis Buffer decreases the sensitivity of some Bead Kits. If a 2-fold final dilution is used, change the titration buffer composition to 50% Lysis Buffer/50% 1X Assay Diluent to ensure accurate quantification. Final dilutions less than 2-fold are not recommended.

Step 1: Prepare Titration Buffer

Quantitative immunoassays are sensitive to buffer composition. Therefore, include the same proportion of Extraction Reagent in all dilutions of standards and samples. The best overall assay performance occurs when lysates are diluted at least 4-fold when incubated with the Capture Beads. Titration buffer as described here (25% Extraction Reagent, 75% 1X Assay Diluent) maintains a 4-fold final dilution of Extraction Reagent in all assay wells.

Note: Prepare fresh titration buffer for each assay.

1. Calculate the total amount of Titration Buffer needed. A minimum of 2000 µl titration buffer is neededto prepare a duplicate standard curve (see *Step 2: Prepare Standard Dilution Series* below). A minimum of ~300 µl titration buffer is needed for each lysate sample that is diluted more than 4-fold final (see optional steps in *Step 3: Prepare Sample Dilutions* on p 12).

   Sample Calculation:

   2 Standard dilution series = 2000 µl

   30 diluted lysate samples = 9000 µl (30 X 300 µl)

   Make at least **11000 µl** titration buffer

2. Prepare the required volume titration buffer by mixing Extraction Reagent from the Cell Extraction Kit and 1X Assay Diluent prepared from the WideScreen™ Reagent Kit. Use a ratio of 25% Extraction Reagent to 75% 1X Assay Diluent. In the example above, take 2750 µl Extraction Reagent + 8250 µl 1X Assay Diluent = 11000 µl Titration Buffer (allowing for additional buffer to account for pipetting error).

Step 2: Prepare Standard Dilution Series

Prepare fresh diluted standards for each assay and use within 1 h.

1. To prepare duplicate 7-point standard curves, label eight microcentrifuge tubes and add 240 µl Titration Buffer to tubes 2–8. See *Step 1: Prepare Titration Buffer* on p 10.
2. Resuspend the appropriate lyophilized Breast Cancer Panel Standards in 120 µl titration buffer for each analyte being tested. These represent 10X Standard solutions. Vortex briefly to ensure all standards are fully resuspended.
3. If conducting a singleplex or using the Breast Cancer Panel I, II, or III Standards Mix, add 30 µl of the standard or the premixed multiplex standards, respectively, to 270 µl Titration Buffer for a final volume of 300 µl. This tube is "Dilution 1" of the standard dilution series. Please refer to Table 3 for the serial dilution series.
4. If conducting a user-assembled multiplex assay, add 30 µl of each of the individual Breast Cancer Panel Standards (10X) being assayed to tube 1. Bring the total volume of tube 1 to 300 µl with Titration Buffer and mix well. This tube is "Dilution 1" of the standard dilution series. Please refer to Table 4 on the next page for an example of the serial dilution series for a user-assembled multiplex.

Notes: *The volume of titration buffer added to reconstitute the 300 µl in a user assembled multiplex will depend upon the number of analytes included in your multiplex. Please refer to Table 4 on the next page for an example.*

The WideScreen Breast Cancer Panel Bead Kits and buffers are not necessarily compatible with other bead kits and reagents sold by Novagen or other vendors. Please refer to Appendix C on p 25 for compatibility of assays found in WideScreen Breast Cancer Panels I, II, and III.

5. Prepare 4-fold serial dilutions from Dilution 1, as follows:
   - Transfer 80 µl from tube 1 to the 240 µl titration buffer in tube 2; mix well.
   - Change tips. Transfer 80 µl from tube 2 to the 240 µl titration buffer in tube 3; mix well.
   - Proceed in similar manner with the serial dilutions through tube 7.
6. The 8th tube contains 240 µl titration buffer only. This will serve as the blank control.

Note: *Refer to* Appendix B *on p 23 for concentrations of the serially-diluted standards.*

Table 3. Serial dilution of singleplex or pre-mixed Breast Cancer Panel I, II, and III standards mix:

Tube/ Dilution	Volume Standard	Volume Titration Buffer	Final Concentration
1	30 µl individual Standard or Breast Cancer Panel I, II or III Standards Mix	270 µl	See Appendix B
2	80 µl from tube 1	240 µl	
3	80 µl from tube 2	240 µl	
4	80 µl from tube 3	240 µl	
5	80 µl from tube 4	240 µl	
6	80 µl from tube 5	240 µl	
7	80 µl from tube 6	240 µl	
8/ BLANK	None	240 µl	0

Table 4. Example of serial dilution of five individual Breast Cancer Panel Standards (user-assembled 5-Plex):

Tube/ Dilution	Vol. Standard	Volume Titration Buffer	Final Concentration
1	5 x 30 µl of each individual Breast Cancer Panel Standard (10X) = 150 µl total volume	150 µl	See Appendix B
2	80 µl from tube 1	240 µl	
3	80 µl from tube 2	240 µl	
4	80 µl from tube 3	240 µl	
5	80 µl from tube 4	240 µl	
6	80 µl from tube 5	240 µl	
7	80 µl from tube 6	240 µl	
8/ BLANK	None	240 µl	0

Note: Individual Breast Cancer Panel Bead Kits can not be multiplexed together in all combinations because of incompatibilities between some assays. Please see Appendix C *on p 25 for further information.*

Step 3: Prepare Sample Dilutions

Notes: Thaw and dilute samples within 1 h of use. Avoid multiple freeze/thaw cycles.

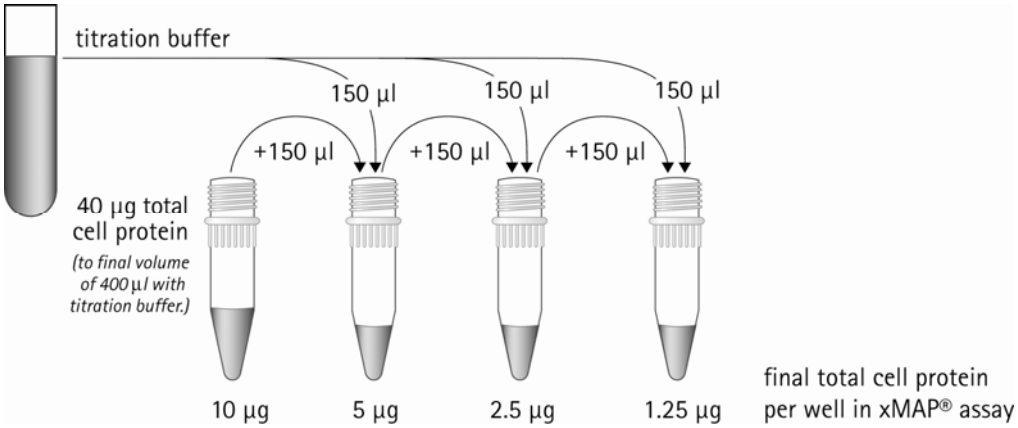
For lysate samples prepared from cells grown in 96-well plates, typical total protein concentrations range from 0.1– 0.5 mg/ml. Therefore, 96-well lysate samples can be diluted directly at the time that the samples are added to the capture beads. Further sample dilutions might not be required (see Step 5: Combine Capture Beads with Analytes *on p 14).*

1. Dilute lysate samples four-fold in 1X Assay Diluent (e.g., 100 µl lysate with 300 µl 1X Assay Diluent). Mix well.

2. Calculate the protein concentration of the four-fold diluted lysate samples based on the protein quantification values previously determined using BCA assay. For example, if the original sample concentration was 1.6 mg/ml, the dilution results in 400 µg/ml.

Note: *If desired, cell extracts can be further diluted to ensure more accurate signal quantification. In this case, follow the optional steps below (Steps 3–5 within this section). A range from 1–10 µg total cell protein per assay well is usually optimal.*

3. Label four centrifuge tubes. In tube 1, mix the four-fold diluted lysate and titration buffer to a final volume of 400 µl and final protein concentration of 100 µg/ml (10 µg/well in the assay). For example, if the four-fold diluted extract has a total protein concentration of 400 µg/ml, mix 100 µl diluted extract with 300 µl titration buffer.
4. If additional dilutions of the extract are desired, prepare three additional 2-fold dilutions of the cell extract, as follows:
   - Add 150 µl titration buffer to tubes 2, 3, and 4.
   - Transfer 150 µl from tube 1 to the 150 µl titration buffer in tube 2 and mix well.
   - Change tips. Transfer 150 µl from tube 2 to the 150 µl titration buffer in tube 3. Mix well.
   - Proceed in similar fashion with the serial dilutions through tube 4.
5. These dilutions will result in 10 µg, 5 µg, 2.5 µg, or 1.25 µg total cell protein per assay well, respectively (refer to figure below).



Step 4: Prepare Capture Beads

Notes: *Prepare diluted Capture Beads within 1 h of use.*

Individual Breast Cancer Panel Bead Kits can not be multiplexed together in all combinations because of incompatibilities between some assays. Please refer to the compatibility chart in Appendix C on p 25 to determine optimal individual bead kit combinations.

1. Calculate the number of test wells needed, allowing ~10% extra for pipetting error.
2. Note the volume of 50X Capture Beads needed per well, based on the assay format. In all cases, this results in approximately 3000 beads per bead region per well.

Assay Format	Vol. Capture Beads (50X) needed
Singleplex (one target)	1 µl per well
User-assembled multiplex	1 µl from each individual Bead Kit per well
Breast Cancer Panel I, II or III multiplex (premixed)	1 µl per well

3. Thoroughly resuspend each vial of Capture Beads (50X) by vortexing for 10 sec, sonicating in an ultrasonic bath for 10 sec (optional), and vortexing again for 5 sec.

4. Each well receives a total of 50 µl diluted (1X) Capture Beads. Determine the total volume of 50X Capture Beads needed per well (refer to table above) and the volume of 1X Assay Diluent needed to bring the total volume per well to 50 µl. Multiply these volumes by the number of test wells to determine the total volumes of each component needed. Refer to the table below for example calculations.
5. Add the calculated volumes of Capture Beads (50X) and 1X Assay Diluent to a microcentrifuge tube. Vortex 3 sec. Protect from light and store at 4°C until use.

Example Calculations: (40 test wells, including 10% extra)

	Singleplex, *or* Breast Cancer Panel I, II or III multiplex (premixed)	**User-assembled Breast Cancer Panel multiplex** (e.g., 5-Plex)
Test wells	40 (44 including 10% extra)	40(44 including 10% extra)
Volume Capture Beads (50X)	1 µl per well	1 µl each bead per well = 5 µl total
Volume 1X Assay Diluent	49 µl per well	45 µl per well
Total Volume Capture Beads (50X)	1 µl beads per well x 44 wells **= 44 µl beads**	5 µl beads per well x 44 wells **= 220 µl beads (44 µl ea)**
Total Volume 1X Assay Diluent	49 µl per well x 44 wells **= 2156 µl**	45 µl per well x 44 wells **= 1980 µl**

Step 5: Combine Capture Beads with Analytes

1. On the 96-well filter plate, tape off wells that are not going to be used for the assay with the provided Plate Sealer (cut to size) or lab tape for future use.
2. Pre-wet the 96-well filter plate for at least 5 min by adding 50 µl 1X Assay Diluent to each well that will be used. With the vacuum manifold, apply gentle vacuum (3 in/76 mm Hg) to filter plate just until liquid aspiration is complete. Do not let the vacuum exceed 5 in/127 mm Hg. Tap filter plate on a paper towel to remove any buffer on the underside.

Notes: *It is critical to remove excess buffer from the underside of the filter plate before adding samples or reagents. Otherwise, samples may wick out of the wells during incubation steps. For the same reason, avoid placing filter plate on an absorbent surface during incubations. See* Considerations Before You Begin *on p 10 for guidelines on using the filter plate vacuum and manifold.*

If well does not drain, use the non-tip end of a fresh 200µl pipet tip to flick the center of the plastic support on the underside of the well, and then reapply the vacuum.

3. Vortex (10 sec) the diluted Capture Beads solution prepared as per *Step 4: Prepare Capture Beads* on p 13. Add 50 µl beads to each well being used.
4. Remove liquid from filter plate by vacuum filtration.
5. To bead-containing wells reserved for the standards, add 100 µl from the standard dilutions (Dilutions 1–7 + blank) prepared as per *Step 2: Preparing Standard Dilution Series* on p 11.
6. To bead-containing wells reserved for analyzing experimental samples, add 100 µl diluted samples prepared as per *Step 3: Prepare Sample Dilutions* on p 12. If additional sample dilutions were prepared (optional), add 100 µl of these dilutions to bead-containing wells.

Note: *If working with samples generated from cells grown in 96-well plates, the four-fold dilution with 1X Assay Diluent is done most easily at this step, rather than as a separate step (see* Step3: Prepare sample dilutions*). To the appropriate bead containing wells, add 75 µl 1X Assay Diluent and 25 µl clarified cell lysate using a multichannel pipet.*

7. Incubate overnight at 4°C on a platform plate shaker (750 rpm). Use aluminum foil to protect filter plate from light.

Notes: *Shorter incubations are possible, but will decrease overall signal strength.*

Make sure that the samples are not incubated below 4°C during this incubation step. This can cause the lysates to acquire a gel-like consistency and adversely affect results.

Step 6: Add Detection Antibodies

Note: Prepare 1X Detection Antibody solution within 1 h of use.

1. Calculate the number of test wells needed.
2. Note the volume of 100X Detection Antibody needed per well, based on the assay format (see table below):

Assay Format	Volume Detection Antibodies (100X) needed
Singleplex (one target)	1 µl per well
User-assembled Breast Cancer Panel multiplex	1 µl from each individual Bead Kit per well
Breast Cancer Panel x-Plex (premixed)	1 µl per well

3. Each well receives a total of 100 µl diluted (1X) Detection Antibody solution. Determine the total volume of 100X Detection Antibodies needed per well (refer to the table above) and the volume of 1X Assay Diluent needed to bring the total volume per well to 100 µl. Multiply these volumes by the number of test wells to determine the total volumes of each component needed. Prepare ~10% extra for pipetting error. Refer to the table below for example calculations.
4. Add the calculated volumes of Detection Antibodies (100X) and 1X Assay Diluent to a microcentrifuge tube. Vortex 3 sec and store at 4°C until use.

Example Calculations: (40 test wells, including 10% extra)

	Singleplex, *or* Breast Cancer Panel I, II or III Multiplex (premixed)	**User-assembled Breast Cancer Panel multiplex (e.g., 5-Plex)**
Test wells	40 (44 including 10% extra)	40(44 including 10% extra)
Volume Detection Antibodies (100X)	1 µl per well	1 µl each Antibody per well = 5 µl total
Volume 1X Assay Diluent	99 µl per well	95 µl per well
Total Volume Detection Antibodies (100X)	1 µl Antibody per well x 44 wells **= 44 µl Detection Antibody**	5 µl Antibodies per well x 44 wells **= 220 µl Detection Antibodies (40 µl ea)**
Total Volume 1X Assay Diluent	99 µl per well x 44 wells **= 4356 µl**	95 µl per well x 44 wells **= 4180 µl**

5. Remove liquid from filter plate by vacuum filtration.
6. Add 100 µl 1X Wash Buffer to each well. Remove liquid by vacuum filtration. Repeat wash and filtration steps twice more, for a total of three washes. Tap filter plate on a paper towel to remove any buffer on the underside.

Note: Do not allow the beads to dry out. Vacuum only long enough to remove all liquid. Add the next solution immediately after tapping filter plate on a paper towel.

7. Immediately add 100 µl 1X Detection Antibody solution to each well.

Note: Turn on the Luminex® System. The lasers require a 30 min warm-up period.

8. Incubate for 1 h at room temperature on a platform plate shaker (750 rpm). Protect from light.

Step 7: Add Streptavidin-Phycoerythrin (PE)

Note: Prepare 1X Streptavidin-PE solution within 30 min of use.

1. Briefly spin the tubes containing the 100X Streptavidin-PE to collect reagent that might be trapped in the tube cap.
2. Calculate the total volume of 1X Streptavidin-PE solution required. 100 µl is needed for each used well.

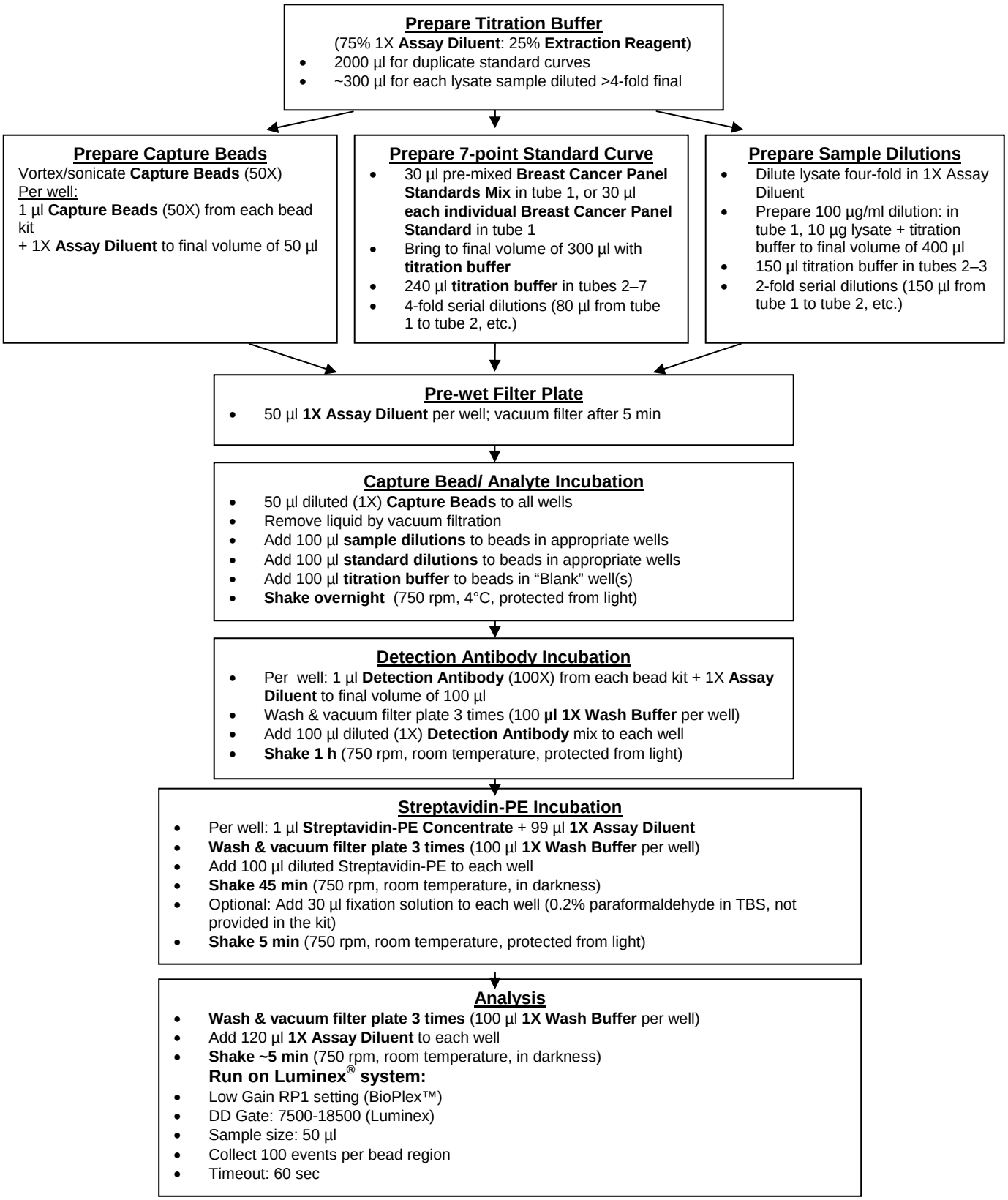
Note: Do not dilute the whole vial of Streptavidin-PE if the entire kit will not be used. Dilute only what is needed based on the number of wells. Allow 10% excess for pipetting error

3. Prepare the calculated volume of 1X Streptavidin-PE solution by diluting Streptavidin-PE Concentrate 1/100 in 1X Assay Diluent. For example, for 40 test wells (44 well including 10% extra), add 44 µl Streptavidin-PE to 4356 µl 1X Assay Diluent to prepare a final volume of 4400 µl.
4. Vortex 3 sec. Protect from light and store at 4°C until use.
5. Wash wells three times with 1X Wash Buffer as described above. After the final vacuum filtration, tap filter plate on a paper towel to remove any buffer on the underside.
6. Immediately add 100 µl 1X Streptavidin-PE solution to each well.
7. Incubate for 45 min at room temperature on a platform plate shaker (750 rpm). Protect from light.
8. Optional: Add 30 µl fixation solution to each well (0.2% paraformaldehyde in PBS, not provided in the kit). Incubate for 5 min at room temperature on a platform plate shaker (750 rpm). Protect from light.

Note: Fixation will improve well-to-well assay reproducibility.

9. Wash wells three times with 1X Wash Buffer as described above. After the final vacuum filtration, tap filter plate on a paper towel to remove any buffer on the underside.
10. Immediately add 120 µl 1X Assay Diluent to the beads in each well. To fully resuspend beads before running samples on the Luminex® System, incubate for 3–5 min on a platform plate shaker. Protect from light.
11. Analyze samples with a Luminex System according to the manufacturer's instructions.

Flowchart for Breast Cancer Panel Immunoassay


Prepare Titration Buffer
(75% 1X Assay Diluent: 25% Extraction Reagent)
2000 µl for duplicate standard curves
~300 µl for each lysate sample diluted >4-fold final
Prepare Capture Beads
Vortex/sonicate Capture Beads (50X)
Per well:
1 µl Capture Beads (50X) from each bead kit
+ 1X Assay Diluent to final volume of 50 µl
Prepare 7-point Standard Curve
30 µl pre-mixed Breast Cancer Panel Standards Mix in tube 1, or 30 µl each individual Breast Cancer Panel Standard in tube 1
Bring to final volume of 300 µl with titration buffer
240 µl titration buffer in tubes 2–7
4-fold serial dilutions (80 µl from tube 1 to tube 2, etc.)
Prepare Sample Dilutions
Dilute lysate four-fold in 1X Assay Diluent
Prepare 100 µg/ml dilution: in tube 1, 10 µg lysate + titration buffer to final volume of 400 µl
150 µl titration buffer in tubes 2–3
2-fold serial dilutions (150 µl from tube 1 to tube 2, etc.)
Pre-wet Filter Plate
50 µl 1X Assay Diluent per well; vacuum filter after 5 min
Capture Bead/ Analyte Incubation
50 µl diluted (1X) Capture Beads to all wells
Remove liquid by vacuum filtration
Add 100 µl sample dilutions to beads in appropriate wells
Add 100 µl standard dilutions to beads in appropriate wells
Add 100 µl titration buffer to beads in "Blank" well(s)
Shake overnight (750 rpm, 4°C, protected from light)
Detection Antibody Incubation
Per well: 1 µl Detection Antibody (100X) from each bead kit + 1X Assay Diluent to final volume of 100 µl
Wash & vacuum filter plate 3 times (100 µl 1X Wash Buffer per well)
Add 100 µl diluted (1X) Detection Antibody mix to each well
Shake 1 h (750 rpm, room temperature, protected from light)
Streptavidin-PE Incubation
Per well: 1 µl Streptavidin-PE Concentrate + 99 µl 1X Assay Diluent
Wash & vacuum filter plate 3 times (100 µl 1X Wash Buffer per well)
Add 100 µl diluted Streptavidin-PE to each well
Shake 45 min (750 rpm, room temperature, in darkness)
Optional: Add 30 µl fixation solution to each well (0.2% paraformaldehyde in TBS, not provided in the kit)
Shake 5 min (750 rpm, room temperature, protected from light)
Analysis
Wash & vacuum filter plate 3 times (100 µl 1X Wash Buffer per well)
Add 120 µl 1X Assay Diluent to each well
Shake ~5 min (750 rpm, room temperature, in darkness)
Run on Luminex® system:
Low Gain RP1 setting (BioPlex™)
DD Gate: 7500-18500 (Luminex)
Sample size: 50 µl
Collect 100 events per bead region
Timeout: 60 sec

Collecting Data and Data Analysis

Data Acquisition

For detailed instructions on the operation of Luminex® Systems, refer to the user guide for your specific software and instrument. General recommendations are given below.

1. Using your Luminex System software, prepare a protocol for the assay you will run. Enter in information for each bead kit target, standards, samples, and controls that will be run. The ranges of final concentrations are shown in Appendix B.
2. Select the bead regions used in the assay. The bead regions used for the Breast Cancer Panel Bead Kits are shown in Appendix B.
3. Format the assay plate, indicating which wells contain which type of analyte.
4. Acquire data using the system settings shown below:

Software	Sample Size	Events per Bead Region	Timeout*	Doublet Discriminator	CAL2 Gain Setting
Luminex® 100 IS™	50 µl	100	60 sec	7500–18500	default
Bio-Plex® Manager™	Default (50 µl)	100	Default (60 sec)	Default (4335–10000)	RP1 Low

** If the time spent acquiring samples needs to be reduced, collect as low as 50 events per bead region, OR adjust the timeout as short as 30 sec.*

Generation of Standard Curves and Quantitation of Experimental Samples

- Standards are available for all of the Breast Cancer Panel Bead Kit (see Appendix B), allowing accurate quantification. Representative standard curves and assay performance information can be found in the Certificates of Analysis for the individual bead kits.
- The 7-point standard curves are plotted using Median Fluorescent Intensity (MFI) as the signal readout (Y-axis) against concentration of standard dilutions (X-axis). Measurements of the blank are useful for assessing background and lower limits of detection. However, it is not necessary to subtract the MFI value of the blank from other measurements, and the blank is generally not plotted as part of the curve.
- Five-Parameter Logistic (5PL) curve fitting is recommended for modeling data. Most ranges of standard curve concentrations are too wide for accurate linear regression analysis. Four-parameter (4PL) equations will often give a good fit, but are not ideal because they assume the standard curve is symmetrical.
- If the signal from an experimental sample exceeds the highest point of the standard curve, the concentration of the unknown should *not* be extrapolated because the non-linear shape of the standard curve cannot be accurately modeled past the last measured point. In this case, samples should be diluted and tested again.

Troubleshooting

Problem	Probable Cause	Solution
Lysate is viscous	Genomic DNA is not digested	Make sure Benzonase® Nuclease was added to Extraction Reagent. Incubate lysate longer, for eg, 30 min. For cell lines with recurring viscosity problems, additional Benzonase Nuclease can be added (available separately).
Leaking wells in filter plate	Wicking due to adherent drops	Tap filter plate several times on paper towel before adding samples or reagents. Do not place filter plate on an absorbent surface during incubations. If wells leaked during data acquisition, it may be possible to reacquire these wells. Blot underside of the wells and add 120 µl/well 1X Assay Diluent.
	Perforation of filter plate membranes	Adjust the vacuum setting to <5 inches (127 mm) Hg. Do not touch membranes with pipet tips.
Filter plate wells not draining under vacuum	Vacuum is too low	Adjust vacuum setting to 3-5 inches (76-127 mm) Hg. Clean rubber seals. Apply fingertip pressure to filter plate to ensure formation of a good seal. Use a plate sealer to cover wells not in use.
	Cell debris clogs membranes	Clarify lysates by centrifugation. Avoid disturbing pellets when removing supernatant. Use the non-tip end of a fresh 200 µl pipette tip to flick the center support on the underside of the well, then reapply vacuum. If lysate protein concentration is high, dilute further before assaying.
Low bead counts during data acquisition	No beads (or wrong beads) in the wells	See solutions above for leaking wells. Verify that the appropriate beads were added at the correct concentration, and that the correct bead regions and wells were selected during acquisition setup.
	Luminex® fluidics system is clogged	Clear system of clogs or air using maintenance steps described in the instrument user manual (sanitize, alcohol flush, probe sonication, etc.). Make sure that the probe height is set correctly. Make sure that beads are in suspension by incubating plate for 3–5 min on the platform plate shaker (750 rpm) immediately before analysis. Microbial growth in buffers can cause beads to stick to the filter plate membrane. Do not use contaminated reagents.
	Timeout limit is set too low	Use the recommended settings for acquisition setup first (50 µl sample, 100 events per bead, 60 sec time out, etc.). However, timeout limit can be set higher, e.g. 75 s.
Data acquisition is slow	No beads in the wells, or fluidics system is clogged	See "Low bead counts during data acquisition" solutions, above.
	Some bead regions being acquired are not in the wells	Make sure that the intended beads were added, and that the correct bead regions and wells were selected during acquisition setup. Attempting to acquire inappropriate bead regions will cause each sample to time out.
Beads are not falling into the gates properly	Beads were not resuspended in 1X Assay Diluent before analysis	The setting of the Doublet Discriminator (DD) gate is buffer-specific. This gate can be adjusted, but 1X Assay Diluent is the buffer recommended for running samples. Other buffers may also cause bead aggregation.
	Beads were exposed to organic solvents	Do not use organic solvents in the immunoassay, as they will damage beads irreversibly.
	Beads are falling outside the bead region gates due to photobleaching	Do not use expired beads. Do not expose the beads to ambient light for >10 min. Avoid intense light.
	Fluidics system is not running properly	Confirm that the waste container is not full, the sheath fluid is not empty, and the SD fluidics module is turned on. Check system calibration using approved calibration beads. Verify correct system pressure. Confirm that the system is free of air or particulate buildup. Follow maintenance steps described in the instrument user manual.

Problem	Probable Cause	Solution
An immunoassay reagent is used up	Solutions were not prepared or used as described in the protocol	Briefly spin tubes to collect reagents that might be trapped in the tube cap. Confirm correct buffer dilutions and use. If additional Wash Buffer is needed, TBST (10 mM Tris pH 7.5, 150 mM, NaCl, 0.05% Tween-20) may be substituted. If additional Assay Diluent is needed, 10 mM Tris pH 7.5, 225 mM NaCl, 0.05% Tween-20, 1% BSA may be substituted. If additional 96-well filter plates are required, we recommend Millipore Cat. No. MSBVN1210. If there is insufficient volume of 100X Streptavidin-PE for your final experiment, making a slightly more dilute working stock (e.g., 0.75X instead of 1X) will not adversely affect results.
High coefficients of variance (CVs) between replicates	Cells grown in 96-well plates show well-to-well variability	To avoid edge effects, don't plate cells in outermost wells of plates. Plate cells uniformly. Add lysis reagents accurately. Do not dislodge adherent cells during pre-lysis wash steps. If necessary, decant (instead of aspirating) liquid and tap plate on paper towels. If cells become less adherent during overnight serum starvation, shorten the serum starvation step to 4 h.
	A gradual drop in signal strength as many samples are read on the xMAP® system	Group samples such that those being compared directly (including replicates) are being read without a long delay in between. Use 0.2% paraformaldehyde in TBS to covalently fix PE to bead surfaces.
	Lysates assayed at different times show assay-to-assay variability	Generate standard curves carefully (using at least duplicate dilutions series) to increase inter-assay precision. Fully resuspend standards and lysate samples by thawing to room temperature and vortexing carefully.
Sample measurements not falling on the standard curve	Dilution of lysate is too low or too high	If values are higher than the standard curve, dilute samples further in titration buffer. Signal strength may be boosted by increasing lysate protein concentration, by lysing cells at a higher confluence, or by using less Extraction Reagent.
	Standard curve and background values increased due to multiplexing	The standard curves of some assays shift slightly upon multiplexing. Therefore, for accurate quantitation, the same multiplex of assays must be prepared when comparing standard curves and experimental samples.
	Target concentration is below detection	Not all analytes are expressed detectably in all cell lines and tissue lysates. Screen additional cell lines. Target expression may be suboptimal in some cell lines or tissue samples. Confirm that antibodies used in the assay recognize target in the species being tested.

Appendix

A: Breast Cancer Panel Kits Ordering and Storage Information

Each Bead Kit contains the following components:

- 100 µl Capture Bead (50X, use 1 µl per test)
- 100 µl Detection Antibody (100X, use 1 µl per test)
- 120 µl Breast Cancer Panel Standard(s) (10x, lyophilized)

Note: Individual Breast Cancer Panel Bead Kits can not be multiplexed together in all combinations because of incompatibilities between some assays. Please see Appendix C *on p 25 for further information.*

Breast Cancer Panel I, 6-Plex			
100 tests	Angiopoietin-2, progesterone receptor, HER-2, PAI-1, IGF-1R, angiogenin	Store at 4°C	72085-3

Breast Cancer Panel II, 5-Plex			
100 tests	EGFR, Fas, VEGFR2, estrogen receptor α, TIMP-2,	Store at 4°C	72086-3
Breast Cancer Panel III, 4-Plex			
100 tests	TIMP-1, uPA, E-Cadherin, IGFBP-3	Store at 4°C	72087-3

Individual Breast Cancer Panel I Bead Kits			
100 tests	Angiopoietin-2 Total Bead Kit	Store at 4°C	72075-3
100 tests	Progesterone receptor Total Bead Kit	Store at 4°C	72081-3
100 tests	HER-2 Total Bead Kit	Store at 4°C	71932-3
100 tests	PAI-1 Total Bead Kit	Store at 4°C	72080-3
100 tests	IGF-1R Total Bead Kit	Store at 4°C	71929-3
100 tests	Angiogenin Total Bead Kit	Store at 4°C	72074-3

Individual Breast Cancer Panel II Bead Kits			
100 tests	EGFR Total Bead Kit	Store at 4°C	71928-3
100 tests	Fas Total Bead Kit	Store at 4°C	72078-3
100 tests	VEGFR-2 Total Bead Kit	Store at 4°C	71933-3
100 tests	Estrogen receptor α Total Bead Kit	Store at 4°C	72077-3
100 tests	TIMP-2 Total Bead Kit	Store at 4°C	72083-3

Individual Breast Cancer Panel III Bead Kits			
100 tests	TIMP-1 Total Bead Kit	Store at 4°C	72082-3
100 tests	uPA Total Bead Kit	Store at 4°C	72084-3
100 tests	E-Cadherin Total Bead Kit	Store at 4°C	72076-3
100 tests	IGFBP-3 Total Bead Kit	Store at 4°C	72079-3

WideScreen™ Breast Cancer Panel I Complete Kit		**72088-3**
1	Breast Cancer Panel 6-Plex, which includes: • Breast Cancer Panel I Capture Beads Premix, 6-Plex • Breast Cancer Panel Detection Antibody Premix, 6-Plex • Breast Cancer Panel I Standards Mix, 6-Plex	Store at 4°C
1	Cell Extraction Kit (see p 5 for components)	Store at –20°C
1	WideScreen Reagent Kit (see p 5 for components)	see p 5 for storage conditions

WideScreen Breast Cancer Panel II Complete Kit		**72089-3**
1	Breast Cancer Panel 5-Plex, which includes: • Breast Cancer Panel II Capture Beads Premix, 5-Plex • Breast Cancer Panel Detection Antibody Premix, 5-Plex • Breast Cancer Panel II Standards Mix, 5-Plex	Store at 4°C
1	Cell Extraction Kit (see p for components)	Store at –20°C
1	WideScreen Reagent Kit (see p 5 for components)	see p 5 for storage conditions

WideScreen Breast Cancer Panel III Complete Kit		**72090-3**
1	Breast Cancer Panel 4-Plex, which includes: • Breast Cancer Panel III Capture Beads Premix, 4-Plex • Breast Cancer Panel Detection Antibody Premix, 4-Plex • Breast Cancer Panel III Standards Mix, 4-Plex	Store at 4°C
1	Cell Extraction Kit (see p 5 for components)	Store at –20°C
1	WideScreen Reagent Kit (see p for components)	see p 5 for storage conditions

Note: The Breast Cancer Panel Bead Kits and reagents are not necessarily compatible with other bead kits and reagents sold by Novagen or other vendors. Please refer to Appendix C on p 25 for compatibility of assays found in WideScreen Breast Cancer Panels I, II, and III.

B: Dilution Series for Generating Standard Curves

The standard curve is used to quantify target proteins found in cell extracts and tissue samples. Standards are recombinant fusion proteins representing all or part of the target proteins.

Notes: *Standard concentrations are assay-dependent. This is because the linear range and lower limit of each assay depends on assay sensitivity. Values shown are the final concentrations in pg/ml.*

Standards supplied with Breast Cancer Panel Bead Kits for individual targets contain only the standard of interest, but can be mixed with other standards (see Appendix C) for multiplex analysis.

Breast Cancer Panel I Standards Mix: Final concentrations in the 4-fold serial dilution of the standards

	Angiopoietin-2	PR	HER-2	PAI-1	IGF-1R	Angiogenin
Mol. Wt [kDa]	**66**	**102**	**96**	**50**	**48**	**14**
Bead Region	**#14**	**#22**	**#72**	**#20**	**#25**	**#13**
	pg/ml	**pg/ml**	**pg/ml**	**pg/ml**	**pg/ml**	**pg/ml**
Dilution 1	10000	3000	20000	20000	100000	200
Dilution 2	2500	750	5000	5000	25000	50
Dilution 3	625	188	1250	1250	6250	13
Dilution 4	156	47	313	313	1563	3
Dilution 5	39	12	78	78	391	0.8
Dilution 6	10	3	20	20	98	0.2
Dilution 7	2.5	0.7	5	5	24	0.1
Blank	0	0	0	0	0	0

Breast Cancer Panel II Standards Mix: Final concentrations in the 4-fold serial dilution of the standards

	EGFR	Fas	VEGFR-2	ERα	TIMP-2
Mol. Wt [kDa]	**68**	**45**	**160**	**66**	**22**
Bead Region	**#21**	**#24**	**#76**	**#17**	**#18**
	pg/ml	**pg/ml**	**pg/ml**	**pg/ml**	**pg/ml**
Dilution 1	20000	2000	100000	50000	30000
Dilution 2	5000	500	25000	12500	7500
Dilution 3	1250	125	6250	3125	1875
Dilution 4	313	31	1563	781	469
Dilution 5	78	8	391	195	117
Dilution 6	20	2	98	49	29
Dilution 7	5	0.5	24	12	7
Blank	0	0	0	0	0

Breast Cancer Panel III Standards Mix: Final concentrations in the 4-fold serial dilution of the standards

	TIMP-1	uPA	E-Cadherin	IGFBP-3
Mol. Wt [kDa]	**20**	**55**	**87**	**29**
Bead Region	**#23**	**#26**	**#15**	**#19**
	pg/ml	**pg/ml**	**pg/ml**	**pg/ml**
Dilution 1	5000	30000	10000	3000
Dilution 2	1250	7500	2500	750
Dilution 3	313	1875	625	188
Dilution 4	78	469	156	47
Dilution 5	20	117	39	12
Dilution 6	5	29	10	3
Dilution 7	1	7	2	0.7
Blank	0	0	0	0

C: WideScreen™ Breast Cancer Panels: Assay Compatibility Chart

Some individual bead-based assays should not be multiplexed together, typically because detection antibodies from one assay will bind non-specifically to capture beads from another assay. This matrix indicates compatibility between all paired combinations of the WideScreen™ Breast Cancer Panel Bead Kits. "Not Compatible" signifies a combination that should not be used together, typically due to unacceptably high background in one of the assays. "Compatible with increased background" combinations may be used with only a minor loss in sensitivity. Compatibility with other bead-based assays from Novagen or other vendors has not been tested.

Note: *This Compatibility Chart identifies cases of non-specific interference between assays, and is not meant to represent assay cross-recognition or target specificity. For information on assay specificity, see the Certificates of Analysis for individual Breast Cancer Panel Bead Kits.*